

Respiratory immunity is an important component of protection elicited by subunit vaccination against pneumonic plague[☆]

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Abstract

Mice were vaccinated with a recombinant fusion protein, rF1-V, by an intramuscular prime followed by an intranasal boost, to evaluate protection against pneumonic plague. Forty-two days after the intranasal boost, the mice were challenged by aerosol exposure to *Yersinia pestis*. Survival after exposure depended upon the dose of rF1-V given i.n. with $\geq 80\%$ survival in the highest dose groups. Pulmonary and serum antibody titers to V were the best predictors of outcome. For vaccinated mice that succumbed to the infection, death was delayed by 1–2 days compared to sham-inoculated controls. Weight loss early after exposure correlated with outcome. Pathology studies indicated a severe, necrotizing bronchopneumonia in vaccinated mice that succumbed to the infection, compatible with a prolonged disease course, while the lungs of sham-inoculated mice had only mild pneumonia, which is compatible with a more rapid disease course. Immunity in the respiratory tract appears to be critical for protection against primary pneumonia caused by *Y. pestis*.

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Keywords: Respiratory immunity; Subunit vaccine; Pneumonic plague

1. Introduction

Yersinia pestis is the causative agent of plague, a disease, which has caused significant mortality in the past, and is still a concern today. Three forms of the disease are recognized; bubonic, septicemic, and pneumonic. Pneumonic plague is characterized by a rapid onset of disease, is highly contagious, and has a high mortality rate if untreated [1–3]. Because

Y. pestis is easily transmitted and infectious by aerosol, its potential to be used as a biological agent is well recognized, and it is listed as a category A select agent by the Centers for Disease Control and Prevention [4]. Licensed vaccines and therapeutics that can protect against this threat are urgently needed.

Vaccines against plague have existed almost since the first isolation of *Y. pestis*. A killed whole-cell vaccine that was used in the US was efficacious against bubonic plague, but not against pneumonic plague [5]. Live, attenuated vaccines protect well against bubonic and pneumonic plague, but there have been adverse events associated with their use [2]. Subunit vaccines, developed from antigenic components of *Y. pestis*, in contrast, can successfully protect against both bubonic and pneumonic plague, and these vaccine products appear to be safe in animal models [6–16].

The F1 and V antigens from *Y. pestis* have the greatest utility for vaccination against plague. The F1 antigen comprises the capsule of *Y. pestis*; it is the primary immuno-

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14. ABSTRACT Mice were vaccinated with a recombinant fusion protein, rF1-V, by an intramuscular prime followed by an intranasal boost, to evaluate protection against pneumonic plague. Forty-two days after the intranasal boost, the mice were challenged by aerosol exposure to Yersinia pestis. Only at the highest doses of rF1-V given intranasally was survival comparable to prior studies of rF1-V given by two intramuscular doses (≥80% survival). Pulmonary and serum antibody titers to V correlated well with outcome. For vaccinated mice that succumbed to the infection, death was delayed by 1-2 days compared to sham-inoculated controls. Weight loss correlated with outcome. Pathology studies indicated a severe, necrotizing bronchopneumonia in vaccinated mice that were euthanized or died, compatible with a prolonged disease course, while the lungs of sham-inoculated mice had only mild pneumonia, which is compatible with a more rapid disease course. Immunity in the respiratory tract appears to be critical for protection against primary pneumonia caused by Y. pestis.					
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gen of the previously used killed whole-cell vaccine [17,18]. However, virulent, F1-negative strains of *Y. pestis* exist, and vaccines based on F1 alone would not, therefore, offer protection against these strains [14]. Because of the failure of the killed whole-cell vaccine to protect against pneumonic plague, there is concern that a vaccine based on F1 alone might not adequately protect against pneumonic plague. The V antigen secreted by *Y. pestis* is important in virulence and it has been postulated that V may be immunosuppressive [19–22]. Vaccination with V alone is sufficient for protecting mice against both bubonic and pneumonic plague [14]. Postulated subunit vaccine candidates for plague use either a combination of F1 and V, or a recombinant fusion of the two [8,9]. Subunit vaccines comprised of other proteins from *Y. pestis* have been evaluated and have demonstrated efficacy, but not to the same degree as F1 and V, either alone or in combination [23].

A question that remains unanswered is the contribution of “local”, mucosal, immunity in the lung to protection against pneumonic plague. Because the previously used, killed whole-cell vaccine offered protection against bubonic, but not pneumonic plague, it suggested that respiratory mucosal immunity could be important in protection against pneumonic plague. However, this has not been formally demonstrated. Herein, we report the results of a study examining the potential for enhancing respiratory immunity against pneumonic plague in mice by using an intranasal booster with rF1-V subunit vaccine.

2. Materials and methods

2.1. Mice

Adult Swiss/Webster mice were purchased from the National Cancer Institute (Frederick, MD) and housed at USAMRIID. Mice were given a commercial mouse food and water, ad libitum.

2.2. Vaccination

Groups of 14 mice were inoculated intramuscularly (i.m.) with 30 µg rF1-V (with aluminum hydroxide) on day 0, and boosted on day 28 with intranasal (i.n.) administration of rF1-V at increasing concentrations in combination with MPL-AF, an aqueous formulation of MPL designed to be given mucosally [24]. For i.n. administration, mice were lightly anesthetized with isoflurane for restraint and no more than 30 µl was placed in the nares. A control group received an i.m. injection of aluminum hydroxide on day 0 and i.n. administration of MPL-AF on day 28.

2.3. Aerosol challenge

Five weeks after the last vaccination, mice were moved into a biosafety level-3 (BSL-3) suite and acclimated for 1

week before being challenged in a nose-only aerosol chamber to an aerosol of *Y. pestis* strain CO92 created by a collision nebulizer, as previously described [9]. Four runs were required to expose all of the animals; even numbers of animals from each group were exposed in each run to ensure dosing was consistent among the groups. Aerosol samples were collected from the all-glass impinger (AGI), attached to the aerosol chamber, and analyzed by plating to determine the inhaled dose of *Y. pestis*. For the four runs, the presented respiratory dose was calculated to be $2.5\text{--}5.2 \times 10^6$ (average 3.9×10^6) of aerosolized colony-forming units (CFU) *Y. pestis* strain CO92 ($\sim 184 \text{ LD}_{50}$).

2.4. Postchallenge monitoring

Mice were monitored daily for 21 days after aerosol challenge. During the first 10 days postexposure, body weight was recorded daily in addition to survival data. Mice that were moribund were euthanized promptly by carbon dioxide overdose.

2.5. Necropsy and histology

Mice that succumbed to disease associated with *Y. pestis* challenge were examined grossly to assess pulmonary disease. After examining the lungs and thoracic cavity in situ, we gently infused 10% neutral buffered formalin (NBF) through the trachea. The pulmonary visceral block was then kept immersed in NBF for 21 days, including a complete change of NBF before passing the tissues out of the BSL-3 facility. Fixed tissues were routinely processed in an automatic tissue processor, embedded in paraffin blocks, sectioned at 5–6 µm on a standard rotary microtome, and mounted on glass microslides for automated staining with hematoxylin and eosin (HE) in a Sakura DRS 601 Slide Stainer (Sakura Finetek USA, Inc., Torrance, CA).

2.6. Collection of blood, bronchoalveolar lavage and nasal lavage

One day before aerosol challenge, four mice from each group were euthanized for assessment of antibody responses. Mice were anesthetized with ketamine hydrochloride and acetylpromazine, injected intraperitoneally, before collection of blood by retro-orbital venipuncture. Mice were then euthanized by an intraperitoneal overdose of barbiturate. Bronchoalveolar lavage (BAL) was then collected by gently infusing and quickly removing 3 ml of PBS through the trachea into the lungs. Using the incision made in the trachea for BAL collection, 1 ml of PBS was flushed retrograde through the nasal passages and collected in a tube.

2.7. Flow cytometry

The protocol used for flow cytometric analysis of the antibody response was adapted from that of McHugh [25].

Serum was serially diluted five-fold in PBAT (PBS/bovine serum albumin/Azide/Tween-20) through six tubes starting at 10^{-2} . Microspheres coated with rF1 or rV were diluted to $10^6/\text{ml}$ and $100\text{ }\mu\text{l}$ of beads was put into each tube with $100\text{ }\mu\text{l}$ of diluted serum. Samples were incubated for 3 h at room temperature in the dark and then washed twice with PBAT. Samples were then incubated for 1 h at room temperature with $100\text{ }\mu\text{l}$ of a 10^{-2} dilution of phycoerythrin (PE)-conjugated goat anti-mouse IgG. Samples were washed twice and resuspended in 0.5 ml of PBAT. Samples were then analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Statistical analysis of antibody titers to percent survival was performed using Sigmaplot 8.0 (SPSS, Inc.)

3. Results and discussion

An initial experiment designed to boost respiratory immunity for protection against pneumonic plague with rF1-V established that a heterologous prime/boost strategy with the prime given i.m. and the boost given i.n. was equivalent to what has been previously reported for two doses of rF1-V given i.m. in terms of survival against challenge[11]. The

experiment was repeated to optimize the dose of rF1-V given i.n. using several doses that were given 0, 30, 40, 50, 75, or $100\text{ }\mu\text{g}$ of rF1-V i.n. on day 28 in combination with MPL-AF. Six weeks after vaccination, samples were collected to analyze antibody titers in sera, the lungs and nasal passages, and mice were challenged by aerosol exposure to $\sim 184\text{ LD}_{50}$ of *Y. pestis* strain CO92.

Fig. 1 and Table 1 show the relationship between antibody titers to V and F1 in sera, lung lavage and nasal passages and survival after aerosol exposure to *Y. pestis*. There was a clear correlation between protection and antibody titers to V in both the sera and lung ($r=0.87$ and $r=0.73$, respectively). Antibody titers to F1 in the sera and lung did not correlate as well ($r=0.67$ and $r=0.64$, respectively) with protection compared to anti-V titers although there appeared to be a minimum threshold after which protection is provided. Titers in the nasal passages to either F1 or V correlated poorly with outcome ($r=0.43$ and $r=0.58$, respectively).

As shown in Fig. 2A, the level of protection afforded by i.n. boosting with rF1-V was dependent upon the dose of rF1-V in the booster dose. At $50\text{ }\mu\text{g}$, 85% of the mice survived challenge. At the 75 and $100\text{ }\mu\text{g}$ levels, the protection was somewhat lower but with the number of mice per group, the

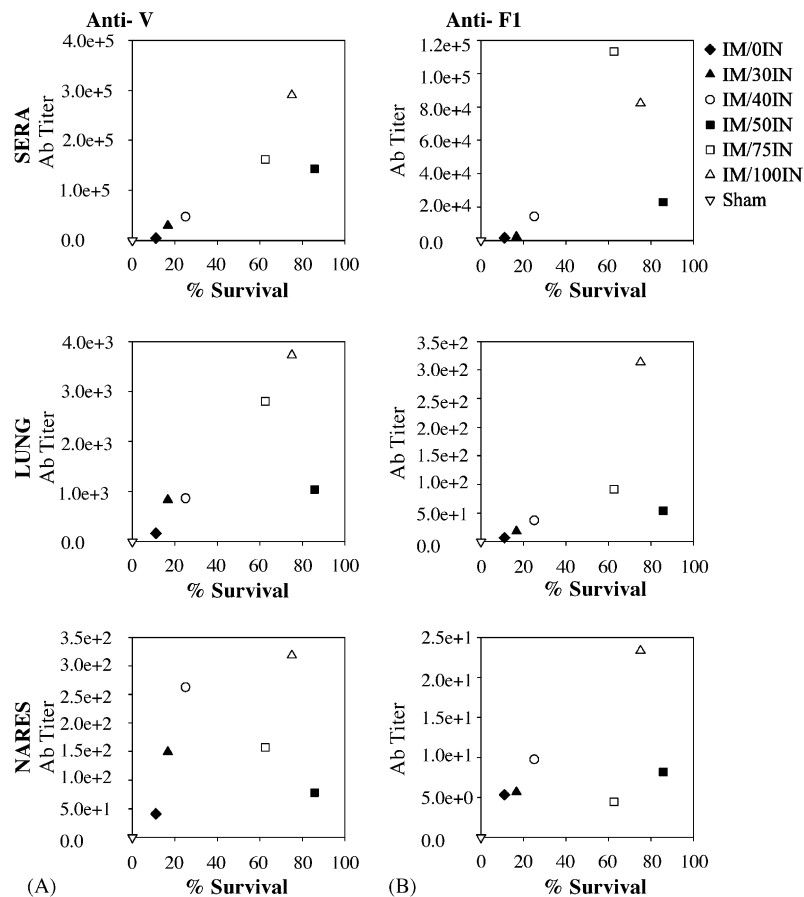


Fig. 1. Relationship of antibody titers in sera, lung, and nasal passages, to F1 and V after vaccination, to protection from pneumonic plague. Dot plots compare the percent survival (x axis) to group mean endpoint titers (y axis) for antibody to F1 and V from sera (top panels), lung (middle panels) and nasal passages (bottom panels). Antibody titers were determined from four animals from each group while percent survival is shown for 10 animals per group.

Table 1

Comparison of antibody responses at different sites and survival after aerosol exposure to *Y. pestis*

Sample	Antigen	i.m./0 i.n.	i.m./30 i.n.	i.m./40 i.n.	i.m./50 i.n.	i.m./75 i.n.	i.m./100 i.n.	Sham
Sera	F1	1535	2270	14285	22875	113343	82204	BLD ^a
	V	4719	29672	47564	143009	161911	290726	BLD
BAL	F1	7	18	37	54	92	314	BLD
	V	167	835	865	1038	2802	3730	BLD
Nares	F1	5	6	10	8	4	23	BLD
	V	41	149	262	78	157	318	BLD
% Survival		11	17	25	86	63	75	0

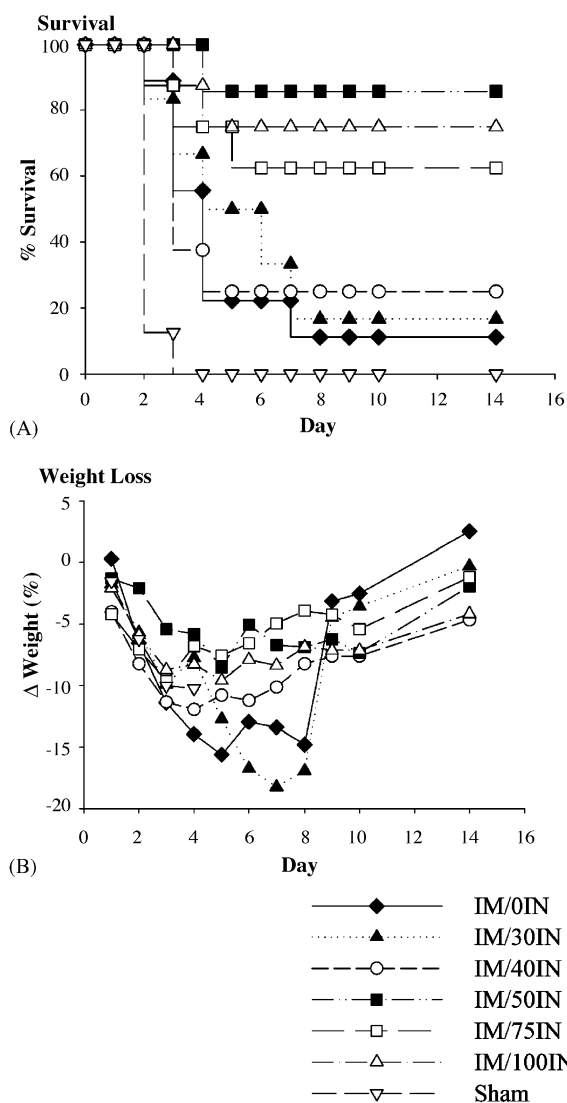
^a Below the limits of detection.

Fig. 2. Survival and weight loss of vaccinated mice after aerosol challenge with *Y. pestis*. Mice were monitored daily for survival and weight beginning just before the exposure and then daily for 10 days after exposure and a final time at 14 days after exposure. Graphs show: (A) the percent survival for each group or (B) the averaged daily percent change in weight (for surviving mice) when compared to baseline pre-exposure weights for mice in each group.

difference in survival at day 14 was not statistically significant. At doses less than 50 μ g, protection was not as good (17% survival across all groups receiving a boost of less than 50 μ g versus 74% collectively for all groups receiving 50 μ g or higher boosts); however, death was delayed when compared to the sham controls. Overall, for sham controls, the mean time to death was 3.1 ± 0.4 days while for vaccinated mice that succumbed to the infection, mean time to death across all the groups was 4.6 ± 1.4 days. The difference in time to death between each of the vaccine groups and the sham controls was significant ($p \leq 0.05$).

In addition to survival, daily body weights were used as an objective measure of illness. Nearly all of the mice, regardless of vaccine dose group, rapidly lost weight in the first few days after aerosol exposure to *Y. pestis* (Fig. 2B), and many had ruffled fur and hunched posture within 3–4 days of exposure. Four days after exposure, all of the sham-vaccinated mice had succumbed to the infection. In the sham group, the disease course was so rapid that the mice, on average, lost only 10% of their body weight before death. In contrast, in the vaccinated groups weight loss was more pronounced, possibly due to the prolonged disease course. By *t*-test, the difference in maximum weight loss between survivors and non-survivors (across all the groups) was highly significant ($p < 0.001$). Fourteen days after exposure, however, the weights of surviving mice in each group had returned to baseline.

Mice that succumbed to *Y. pestis* were necropsied and the lungs removed for histopathology. Gross examination of the lungs clearly revealed differences between the sham-vaccinated controls and vaccinated mice that were euthanized or died. Lungs of sham-vaccinated mice appeared normal; however, lungs from vaccinated mice that succumbed had multiple areas of congestion and hemorrhage and were only partially collapsed.

Microscopy corroborated gross findings. Lungs from sham-immunized mice that succumbed had mild, multifocal, acute bronchopneumonia, which was characterized by the presence of neutrophils, macrophages, plague bacilli, and hemorrhage in small airways and alveoli, with minimal perivascular edema. (Fig. 3A and B). In contrast, the lungs of those vaccinated mice that succumbed were characterized by severe, necrotizing bronchopneumonia, with vascular necrosis, alveolar and small airway edema and fibrin deposition,

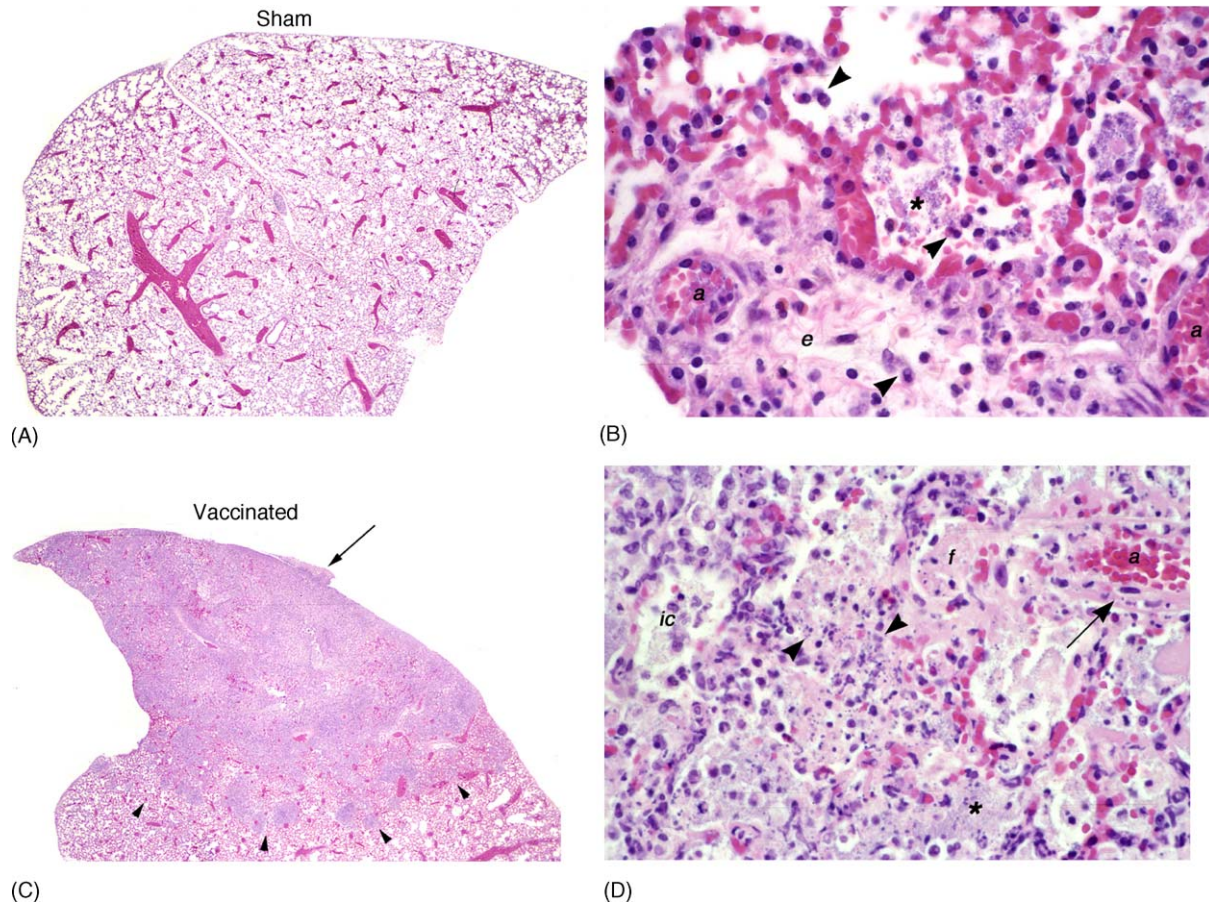


Fig. 3. Lung from sham- and rF1V-inoculated mice that died or were euthanized after aerosol exposure to *Y. pestis*. (A) Lung from a sham-inoculated mouse appears within normal limits. 20 \times , HE. (B) High magnification of lung from a different sham-inoculated mouse shows acute, focal, pneumonia (alveolitis). Note alveolar macrophages and neutrophils (arrowheads), alveolar hemorrhage, perivascular edema, and myriad plague bacilli (*); bronchiolar artery (a). 400 \times , HE. (C) Lung from a rF1-V-vaccinated mouse shows extensive bronchopneumonia involving the apex of a lobe. Note the discrete foci of pneumonia demarcating affected from less affected (arrowheads). Also note thickening of the pleura (arrow) representing extension of the inflammation to the visceral pleura. 20 \times , HE. (D) High magnification of lung from a different rF1-V-vaccinated mouse showing fibrinous, necrotizing pneumonia. Note cellular debris (arrowheads), alveolar fibrin deposition (f) and edema (e), macrophages and degenerating neutrophils (ic), bronchiolar artery (a), vascular necrosis (arrow), and myriad plague bacilli (*). Original magnification 400 \times , HE.

Table 2

Salient pathologic changes evident in lungs of F1-V- and sham-inoculated mice that died or were euthanized after aerosol exposure to *Y. pestis*

	i.m.	i.m./30 i.n.	i.m./40 i.n.	i.m./50 i.n.	i.m./75 i.n.	i.m./100 i.n.	Sham
Pneumonia, necrotizing ^a	4	4	4	4	4	4	2
Bronchiolitis	+	+	+	+	+	+	—
Plague bacilli	+	+	+	+	+	+	+
Alveolar macrophages	+	+	+	+	+	+	—
Neutrophils	+	+	+	+	+	+	—
Vascular necrosis and/or thrombus	+	+	+	+	+	+	—
Leukocytoclastic vasculitis	+	+	+	+	+	+	—
Fibrin	+	+	+	+	+	+	—
Hemorrhage	+	+	+	+	+	+	+
Alveolar or interstitial edema	+	+	+	+	+	+	—
Pleuritis	+	+	+	+	+	+	+
Mediastinitis	+	+	+	+	+	+	—
Mediastinal lymphadenitis	+	±	±	ne	±	—	+
Congestion	+	+	+	+	+	+	+
Number of mice	8	5	6	1	2	2	5

“+” present; “—” not evident; “±” intermediate phenotype; “ne” not examined.

^a Number shown is a score indicative of the severity of the bronchopneumonia with 1 being clear and 4 the most severe.

macrophages, viable and degenerate neutrophils, and myriad plague bacilli. (Fig. 3C and D) (Table 2).

This study was designed to examine whether protection against pneumonic plague could be enhanced by i.n. booster, with a subunit vaccine, after an i.m. prime with the same vaccine. Because airborne transmission of *Y. pestis* causes primary pneumonic plague, it is logical that an alternative route of vaccine administration aimed at enhancing respiratory mucosal immunity would enhance protection. Furthermore, the i.m. prime/i.n. boost regimen was examined here because it was previously shown to be as efficacious as two doses given i.m. (data not shown). In addition, delivery of a vaccine boost by a needle-less delivery system could be more easily administered outside of traditional medical settings. To a certain extent, this approach was not successful; i.n. vaccination using rF1-V required substantially higher amounts to achieve the same level of protection seen when rF1-V, with Alhydrogel, was given by two i.m. injections [9]. MPL-AF is an aqueous formulation of a detoxified endotoxin shown previously to act as an adjuvant when given by i.n. administration [26]. It is thought to induce Th1 type immune responses; we did not address the isotype of the IgG response in these studies although it is possible that it could have played a role in the protection seen. Future studies will compare the effect of utilizing other adjuvants for i.n. vaccination using rF1-V, including those that would stimulate a Th2 type response.

Antibody is an important component of protection against *Y. pestis*. Previous studies have shown that passive immunization with antibodies to either F1 or V was sufficient to protect mice against bubonic and pneumonic plague [27,28]. In this study, F1 titers did not correlate well with protection against pneumonic plague. This is not particularly surprising as F1 is thought to be the primary immunogen of the killed whole-cell vaccines that protected well against bubonic plague but not pneumonic plague [17,18]. In contrast, both the serum and lung antibody titers to the V antigen correlated well with protection in this study. The V antigen is critical to virulence, and vaccines consisting solely of V protect mice [14]. Other studies looking at the antibody response to F1 or V in mice have primarily focused on the response in the sera; few have looked at antibody levels in the respiratory tract. Williamson et al., who used a combination of the F1 and V subunit vaccines, found low titers of antibody in the lung compared with significantly higher levels in sera for both F1 and V [8]. They suggested that because of the higher levels of specific antibody found in the sera, serum titers were a better correlate of immunity than antibody from the lung. Glynn et al. recently reported that using a “heterologous” boosting regimen (i.n. prime and subcutaneous or transcutaneous boost of rF1-V in combination with LTR192G) elicited high titers to the rF1-V fusion protein in both sera and lung lavage of mice [6]. However, separate titers to F1 and V were only assessed in sera but not the lung and protection against challenge with *Y. pestis* was not reported. Cell-mediated immunity to *Y. pestis* is also likely to be important for vaccine-mediated protection against plague, however, the exact nature and role of CMI

in protection is not clear at this time. Studies in mice have suggested an essential role for γ -IFN and TNF- α , which may be suppressed by V antigen in naive mice [21,29]. Peptides made of the V antigen from *Y. pestis* are recognized by murine T cells and vaccination with V peptides induced γ -IFN production by CD4⁺ T cells and were sufficient to protect in vivo against challenge with *Y. enterocolitica* [30].

Our findings indicate that in the absence of specific respiratory or systemic immunity, inhalational exposure to *Y. pestis* led to a rapid and lethal disease in Swiss/Webster mice. The fulminating disease course suggests that death was from endotoxin-mediated shock in the sham-vaccinated mice. Vaccinated mice that mount both a systemic and a respiratory immune response are protected. The changes in disease course in the vaccinated mice that did succumb to infection (increased weight loss and longer time to death), as well as the pulmonary disease seen in the lungs suggest that in these mice dissemination of *Y. pestis* was prevented leading to development of a lethal pneumonia. We believe that these results could be explained by a systemic immune response sufficient to prevent dissemination combined with a respiratory immune response insufficient for clearance from the lung. The data reported here do suggest that respiratory immunity is important for protection against inhalation of *Y. pestis* and strategies to enhancing antibody levels in the respiratory tract could improve efficacy of proposed vaccines.

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